# SOME FACTORS AFFECTING CHROMATOGRAPHIC SEPARATION OF VITAMIN A ON ALUMINA

by

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# INTRODUCTION

Chromatographic separation was introduced by Michael Tswett (1906) early in the twentieth century. It is a method which permits the separation of materials on a single adsorbent. Tswett was a botanist, primarily interested in the study of natural pigments. In 1931, Kuhn, Winterstein and Lederer resolved carotene into several components by chromatography, and fulfilled Tswett's prophesy that "very likely carotene is not a chemical entity but a mixture of two or more homologues which it may be possible to separate from each other by means of the adsorption method". The vitamin A activity of most vegetables and fruits is due to the β-carotene content; α-carotene, γ-carotene and cryptoxanthin appear in much smaller quantities.

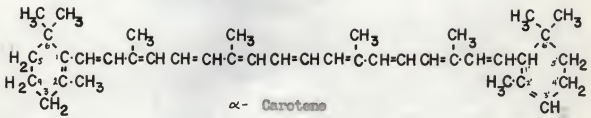
The term chromatography came from the Greek words "chroma" or "chromatos" meaning color, and "graphy" referring to description. Chromatography, as described by Williams (1946), is a process for separating substances by passing their solution through a column of adsorbent. The solution containing the compounds to be separated is added to the top of the column. Components are adsorbed differentially on the column, depending on the affinity of the adsorbent for the component. By using suitable solvents, the adsorbed components are eluted separately. Chromatography often provides an easy method for analysing complex as well as simple mixtures. It is not only applicable to the separation of colored substances but also colorless substances. The chromatographic method is good for the routine analysis of complex natural mixtures where the large number of unknown factors make ordinary analytical methods difficult. It is therefore being used in the analysis of such important mixtures as foods, drugs,

dyes and oils. By chromatography one can determine whether a substance is homogeneous or not. Chromatography also can be used to find whether a vitamin is contaminated. Impurities can be separated from the vitamin in one or more of the following ways: (1) Impurities pass rapidly through the column ahead of the vitamin. (2) Impurities elute at different rates and so can be separated. (3) Impurities are adsorbed tightly so that, for example, when carotenoids are eluted, the impurities are held at the top of the column. When estimating vitamin A, the solution must be free from oxidized products, carotenoids and sterols, as these interfere with the Carr-Price test (Karrer and Jucker, 1950).

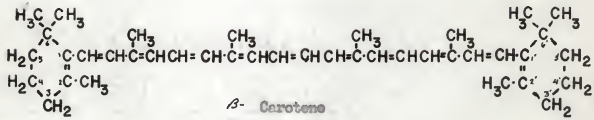
According to Karrer and Jucker (1950), cis-trans isomerism occurs in the carotenoid series, but natural carotenoids are always present in trans-configuration. Carotenoids can be isomerized to the cis form by artificial means in the laboratory. Some of the methods are: (1) refluxing with organic solvent, (2) melting the crystals, (3) treating with acid. Some isomerization is brought about under natural processing conditions, such as dehydration. The cis isomers have lower biological activity than trans isomers (Moore, 1957). The isomerized products can be separated by chromatography under special conditions, but it is a difficult process.

Carotenoids are present in almost all green and yellow vegetables. There are more than 50 known carotenoids of vegetable origin. Some of the common carotenoids are β-carotene, α-carotene, γ-carotene, cryptoxanthin, zeaxanthin and lutein. β-Carotene contains two β-ionone rings. α-Carotene has one β-ionone ring and one α-ionone ring. γ-Carotene is composed of one intact β-ionone ring and one open ring.

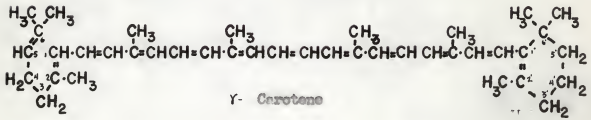
The structures of some of the carotenoids are given below:



α- Carotene



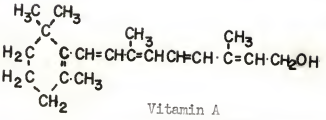
β- Carotene



γ- Carotene

Cryptoxanthin differs from β-carotene in having one β-ionone ring and one hydroxylated β-ionone ring, instead of two β-ionone rings. Zeaxanthin has two hydroxylated β-ionone rings, and lutein contains one α-ionone ring and one β-ionone ring, both hydroxylated. The hydroxyl groups are present in the 4 or 4' positions of the rings (Moore, 1957).

The observations of McCollum and Davis (1913, 1914, 1915) and of Osborne and Mendel (1913, 1915), who found that a factor occurring in certain fats was essential for the growth of rats, led to the discovery of vitamin A. The following structure of vitamin A was introduced by Karrer et al. (1931). The formula also was confirmed by Heilbron et al. (1932).



Vitamin A

In the development of the A. O. A. C. method for vitamin A in mixed feeds (Methods of Analysis, A. O. A. C., 1960), it was found that occasionally results were affected by unknown factors. The objective of the present study was to make an investigation of various factors which during the chromatographic step might effect results of the determination of vitamin A and carotene in feeds. Among the factors that it was decided to investigate are (1) temperature, (2) use of different varieties of alumina, (3) moisture content of the alumina, (4) presence of unsaponifiable extract of

fat, (5) light, and (6) pretreatment of adsorbent with air.

## TYPES OF CHROMATOGRAPHY[1]

Some of the most important types of chromatography are, (1) Adsorption Chromatography, (2) Partition Chromatography, and (3) Ion Exchange Chromatography.

### Adsorption Chromatography

In adsorption chromatography the solution containing substances to be separated is passed through a suitable adsorption column, where the substances are adsorbed differentially, depending upon their affinities for the adsorbent. The substance which has the highest affinity remains adsorbed nearest the top of the column, displacing substances with lower affinities. Thus each substance with a lower affinity is adsorbed in the next lower zone. The different substances which are adsorbed in different zones can be eluted separately by using suitable solvents. As these substances have different rates of elution they come out in different fractions of the eluate. Sometimes it is convenient to use solvents of gradually increasing eluting power for washing out the bands.

Adsorbed substances can be removed from the column by two different methods: mechanical separation and solution, and flowing chromatogram.

Mechanical separation follows the procedure developed by Tswett. If the different adsorbed zones are not distinct, the zones are separated

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[1]The following discussion of chromatography is based on materials in the publications by Lederer and Lederer (1955), Williams (1946, 1950), and Zechmeister (1948). These publications may be consulted for further information of the subject.

from one another by pouring solvent through the column. Then the column is dried and pushed out. The zones bearing different pigments are separated with a knife. From the different zones the pigments are dissolved with solvents. Tswett used calcium carbonate as adsorbent for separation of natural pigments. By washing with petroleum ether he separated the zones before dissecting the column.

The flowing chromatogram is an improvement in Tswett's technique. As colorless compounds are difficult to separate by Tswett's method, the flowing chromatogram was developed by Reichstein (1938). In this method the adsorbed substances are eluted by single or mixed solvents and are separated due to their different elution rates. Reichstein (1938) separated terpenes and steroids by using a series of solvents consisting of pentane, benzene-pentane (1 : 4), benzene-pentane (1 : 1), benzene, ether and acetone. In the present work, 4 percent acetone-in-hexane, and 15 percent acetone-in-hexane were used for eluting carotene and vitamin A, respectively, from the alumina column.

Various adsorbents are used for adsorption chromatography, such as charcoal, bone meal, magnesia, alumina, calcium carbonate, calcium hydroxide, dicalcium phosphate, calcium oxalate, zinc carbonate, and silica gel.

Some of these solid adsorbents are suitable for chromatography of carotene and vitamin A. They are alumina, magnesia, calcium hydroxide, calcium carbonate and bone meal.

## Partition Chromatography

The principle of partition chromatography is different from that

of adsorption chromatography. In this case the separation of a mixture results from being distributed in two immiscible solvents. A great number of consecutive partitions occur between two solvents and ultimately the substances are separated.

Partition chromatography can be classified into two types: column chromatography and paper chromatography.

In column chromatography, an inert column packing is used as support for the stationary phase. The inert column packing is mixed with a suitable solvent, and becomes coated with this solvent. The solvent is known as the stationary phase. Thus the column actually is composed of this mixture. The substances to be separated are dissolved in another suitable solvent, the mobile phase, which is passed through the column. When the mobile phase with its contents is passed through the column, the substances become distributed in the two liquid phases according to their solubilities, and the dissolved substances are separated ultimately into different zones. The zones can be eluted separately with suitable solvents. In developing this method, Martin and Synge (1945) used silica gel containing bound water for separating amino acids dissolved in chloroform containing a small amount of butanol.

Numerous substances can be separated by use of various types of solid supports for the stationary phase and different developing solvents. Cellulose columns saturated with water can be used for separating sugars dissolved in water saturated with n-butanol. Silica gel is one of the most important solid supports used for purification of penicillin. Starch columns are used for amino acid separations.

In paper chromatography a paper strip is used as support for stationary solvent phase (usually water). Paper chromatography may be either

one dimensional or two dimensional. In one dimensional work, a drop of mixture to be separated, dissolved in water or other suitable solvent, is placed near the end of a strip of paper. The edge of the paper then is dipped in a suitable solvent. As the solvent ascends the paper, the substances present in the mixture move and become distributed between the two liquid phases, each substance of the solute mixture moving at different rates, depending upon its nature. Thus they are separated. Then the paper is removed, dried and spotted with suitable reagent. The individual substances may be identified by their Rf values, which is defined as the ratio of the distance moved by a solute to the distance moved by the solvent.

For two-dimensional chromatography, rectangular sheets of paper are used. First the one-dimensional chromatogram is developed along one side of the paper, then the paper is turned through $90^{\circ}$ and the end dipped into a second solvent. The chromatogram again is developed. Substances which are similar and difficult to separate, such as leucine and isoleucine, often can be separated by this technique.

Paper chromatography also can be used in the descending type of chromatogram. In this case the paper is suspended so that the solvent descends through the paper instead of ascending it. The rest of the procedure is the same as in the case of ascending chromatography. Paper can be used as an adsorption media as well as support for the partition column. Sometimes in chromatographic separation, a combination of partition and adsorption occurs and it becomes difficult to define exactly what processes are in operation.

In paper chromatography, generally an organic solvent partially

miscible with water, e.g., phenol, n-butyl alcohol, or pyridine gives the most satisfactory separations. Paper chromatography is mostly used for identification of amino acids, dyestuffs, fatty acids, etc.

## Ion Exchange Chromatography

Some solid substances have properties of exchanging ions with other ions in a solution passed through them. Zeolites have such exchange properties. They remove calcium and magnesium ions from solution in exchange for sodium. Water is purified by this method. In ion exchange chromatography, substances having ion-exchange properties are used as the column. Adams and Holmes (1935) introduced synthetic resins for this purpose, because ions in them exchange more easily than those in natural resins. These resins can exchange either cations or anions. The method originally was developed to separate rare-earth metals, but it has applications in other fields also.

In ion exchange chromatography a suitable resin is selected for the column. The substances to be separated are dissolved in a solvent and are passed through the column. The ions of the solute are removed by the column in exchange for the ions present in the resins. Then elution of the ions picked up by the resin is carried out by displacement with a more strongly adsorbed ion or highly concentrated solution of other ions. The concentrations of adsorbed substances on the resin also can be decreased by addition of a complexing agent. Busch *et al.* (1952) used Dowex-1 for the separation of acids of the citric acid cycle by eluting with increased concentrations of formic acid.

As example of the use of this method, amino acids can be separated on the cation exchanger Amberlite IR-C-50. Purine and pyrimidine bases

can be separated on the cation exchanger Dowex-50 or anion-exchanger Dowex-A1. Ion exchange resins are employed in many industrial processes, such as water softening, sugar refining, metal concentration, etc. This method cannot be used for the separation of vitamin A, which is lacking in the ion exchange properties necessary for this method.

Some other important chromatographic methods, such as gas chromatography, electrophoresis, etc. may be added to the above list; but, since these have no direct relationship to the present investigation, they are not reviewed here.

## REVIEW OF LITERATURE ON CHROMATOGRAPHIC ALUMINA FOR VITAMIN A AND CAROTENE ANALYSIS

### Types of Alumina

According to Mellor (1924), alumina occurs in two distinct forms. α-Alumina is ordinary crystalline alumina represented by corundum (hexagonal prism); β-alumina consists of hexagonal crystals containing groups of overlapping triangular plates. Tertian (1950) studied the structure of γ-alumina, observing that γ-alumina had the same structure as corundum (hexagonal).

Mellor (1924) observed that when pure alumina melted and slowly cooled it changed to the β-alumina. He also observed that presence of 0.5 percent magnesia facilitated formation of β-alumina, whereas presence of lime or silica facilitated formation of the α-variety. Yamauchi (1943), and Zhdanov and Razmanova (1951) also studied changes in form of alumina with changes in temperature. Smith and Beeck (1948) prepared β-alumina by treating γ-alumina with alkali. They observed that γ-alumina was unstable

thermodynamically and changed to α-alumina.

Moeller (1952) described three types of alumina: (a) α-alumina (corundum), melting point 2030° C, boiling point 2980° C, specific gravity 3.99. (b) β-alumina ($Na_2O \cdot 11\ Al_2O_3$). (c) γ-alumina, Sp. gr. 3.42 - 3.64. Moeller (1952) stated that α-alumina is insoluble in water and very resistant to attack by aqueous acids. The γ-form is hygroscopic and dissolves in acids.

According to Smith and Beeck (1948), γ-alumina is unstable thermodynamically and changes to α-alumina, β-alumina is stable thermodynamically. β-alumina is used for catalytical purposes, specially for high temperature reactions such as cracking and dehydrogenation of oil. α-alumina is mostly used for chromatography (Wohlleben, 1958).

## General Principles of Adsorption and Activity

In describing the general principles of adsorption and activity, Williams (1946) states that as a mixture containing different substances passes through a column, the substances at first are adsorbed. Then those substances of higher affinity for the adsorbent displace those of a weaker affinity. The ultimate result is that the substances become arranged in order of their adsorption affinities on the column. The adsorbed substances may be eluted separately by suitable solvents.

The activity of an adsorbent is measured as the affinity for different substances. Alumina was graded 1 to 5 by Brockmann and Schodder (1941) according to the behavior with respect to the organic dyes, (a) P-methoxyazobenzene, (b) Sudan Yellow, (c) Sudan Red, (d) P-aminoazobenzene, (e) P-oxyazobenzene, respectively. The alumina (Merck no. 1089) was heated to

red heat in an open vessel for 4 to 6 hours, with constant stirring to remove moisture. Then it was cooled in an evacuated desiccator. This process gave the most active alumina, grade 1. Grades 2 to 5 were prepared simply by exposing grade 1 to a damp atmosphere. It appeared that the adsorptive activity was inversely related to the amount of water taken up.

To obtain alumina grades 1 to 5, using Woelm alumina, addition of the following amounts of water is required[1]:

| Water addition as percent of the adsorbent. | Activity grade. |
|---|---|
| 0 | 1 |
| 3 | 2 |
| 6 | 3 |
| 10 | 4 |
| 15 | 5 |

Alumina is one of the most useful adsorbents for chromatography, because it is insoluble in both water and organic solvents, and the activity can be controlled and standardized (Williams, 1946).

## Preparation of Alumina

Aluminum oxides of various adsorptive activities were prepared by Brockmann and Schodder (1941) by heating Merck's Technical $Al(OH)_3$ to a strong red heat and partially deactivating by shaking for shorter or longer periods with moist air.

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[1] M. Woelm. Eschwege. Manufacturers brochure, supplied by Alupharm Chemicals, New Orleans, La.

Dupont et al. (1948) prepared alumina for chromatography by the following method: Technical $Al_2O_3$ hydrate was calcined at 500° for one hour. It was soaked in conc. HCl for 2 to 3 hours. The mixture was washed with water till neutral, and dried at 105°. When apparently dry it was tested by the Brockmann and Schodder method for standardization.

Aoyama and Kawaguchi (1951) standardized alumina using the tomato pigments as test substances. They observed a sharp separation of carotene and lycopene when a superior sample of alumina was used, whereas an inferior sample of alumina did not separate carotene from lycopene.

Harris and Sing (1955) prepared alumina by treating $AlCl_3$ or $Al_2(SO_4)_3$ with NaOH at pH 5. The solid was separated by centrifugation, washed with water and dried. They studied the surface properties of the alumina precipitates formed in this manner. Murata and Kukudo (1946) prepared adsorbent - active alumina by treatment of alumina with concentrated sulfuric acid. The two substances were mixed to a paste, then treated with $K_2Cr_2O_7$ and water. Finally the mixture was washed with water, dried and calcined to make adsorbent - active alumina.

## Properties of Alumina

MacIver et al. (1958) observed that hydrated alumina did not lose weight below 145° at atmospheric pressure. Above that temperature decomposition started with loss of water, and at 200° the trihydrate was completely decomposed. The porous $Al_2O_3$ still retained 8 percent water, which was given up slowly as the temperature was increased, indicating that it was held by adsorption. MacIver et al. measured adsorption of propane and butane on alumina using a volumetric procedure and radiotracer technique.

The adsorption of propane and butane on alumina decreased continuously with increase of temperature from 100° to 350°. Decomposition occurred by heating above this temperature.

Newsome and Derr (1945) observed that the autoclaving of alumina, activated at 300° to 500°, in water or steam increased the adsorptive capacity for water at low humidities and decreased the loss of adsorptive capacity on repeated reactivation. Adsorbent alumina prepared by precipitation from aquous solution drying contained 2 to 15 percent water after heating at 300° to 500°. Heating that alumina with water under pressure increased the density and converted a proportion to monohydrate. Five percent of the added water was held in such form that it was not driven out in normal heating.

According to Giovanni Venturello (1943-44), the chromatographic adsorption of various compounds by alumina was due to the presence of impurities, as sodium carbonate and sodium bicarbonate. It was reported by Siewert and Jungnickel (1943) that the alkaline reaction of commercial alumina (Kahlbaun and Merck) was due to the presence of sodium carbonate and sodium bicarbonate, and that Na-aluminate was present in only very subordinate amounts. When pure water was passed through a 100 g. column of alumina (Merck), the filtrate was at first alkaline but ceased being so after one litre of water passed through. The alumina thus washed alkali-free no longer had any appreciable adsorptive effects on cations, as shown by parallel experiments using the original and washed alumina, respectively, with alkaloid salts.

Schwab and Ghosh (1939), cited by Siewert and Jungnickel (1943), concluded that the alkaline reaction of alumina alone does not suffice to

explain its adsorption phenomenon, especially the adsorption series of the cations studied by them. They believed, in contrast to reports of Siewert and Jungnickel (1943), that commercial alumina had exchange properties possible because of the presence of Na-aluminate.

The chromatographic activity K (the affinity of adsorbent for dissolved substances), of alumina, obtained by different treatments of technical alumina, was determined by Kutsakov (1958) as the amount of methylene blue adsorbed from a solution of 0.1 gm of the powder. Rapid calcination at 900° produced alumina with the highest K. The fineness of the particles, between 48 and 188μ, or pretreatment of the powdered alumina with solutions of sodium silicate, affected K very little. The treatment with mineral acids reduced K, and washing with water until neutral to methyl orange reduced K for some inorganic cations to zero.

The degree of activity of alumina was determined by Valentin and Kirchubel (1951) by passing a 0.1 percent petroleum ether solution of Sudan Red through it and measuring the width of the colored zone, which was inversely proportional to the activity of the adsorbent.

Regarding water adsorption by active alumina, Wohlleben (1958) stated that though storage had no effect on chromatographic behavior of alumina, a minimum storage time of two hours should be allowed after the addition of the required amount of water to alumina of activity grade 1 for deactivation before it is used. The quantity of water used for the partial rehydration ought to be considered when using the Karl-Fisher titration method for determining the chromatographic activity of alumina, as a certain percentage of water cannot be recovered, which depends upon the percentage of water added. Adsorption of vapours on alumina (Mallinckrodt Chem. Co.) was studied

by Munro and Johnson (1926) who showed that alumina adsorbed a greater amount of water than any other vapour. Earlier, Munro and Johnson (1925) studied adsorption of some 27 substances, including hydrocarbons, alcohols, esters, ether, halogen derivatives, etc., and showed that adsorbed vapours can be removed easily by raising the temperature of the $Al_2O_3$ a few degrees above the boiling point of the vapour and at the same time passing air through the adsorbent. Alumina cannot be used for adsorption of vapours that decompose in contact with it.

## Carotene

Karrer and Schopp (1932) apparently first proposed the use of an alumina column to separate β-carotene from vitamin A. Few studies, however, have been undertaken using alumina for chromatographic separation of carotene.

It was observed by Gillam and El Ridi (1935) that when β-carotene of grass in petroleum was passed through alumina, there was a single zone in the first adsorption. When this β-carotene was eluted by petroleum ether and was passed through the column a second time, two zones were observed, one having adsorption maxima characteristic of β and the other of α-carotene. Eluation and readsorption of either of the two gave another separation into two colored zones.

Parrish (1959) with his collaborators compared alumina (pretreated according to A.O.A.C. method) and Woelm alumina for the chromatographic separation of carotene in the vitamin A study. The results were generally similar, except that Woelm alumina gave slightly higher values.

## Vitamin A

For the estimation of vitamin A in whale liver oil, which had kitol and other interfering substances not removable by saponification, a chromatographic technique was developed by Gridgeman et al. (1948). This method is based on the fact that the main components of the unsaponifiable fraction of whale liver oil were selectively adsorbed on weakly active alumina in the following order: anhydro vitamin A < oxidized vitamin A < vitamin A alcohol < kitol < sterol. The technique consisted of depositing the material on the column from a non-polar solvent and developing and eluting with solvents of progressively increasing polarity. The elute was collected fractionally and Carr-Price reagent (Carr-Price, 1926) was used to identify vitamin A.

Interfering adsorbing substances, originating from the unsaponifiable extract of margarine containing vitamin A, were eliminated by Boldingh and Drost (1951) through use of double chromatography on two adsorbents in series. The top chromatographic tube contained $Al_2O_3$ and the bottom tube contained $Al_2O_3$ treated with 10 percent NaOH solution and dried at 100° C.

An improved technique of chromatographic separation of vitamin A was claimed by Müller (1944). Results obtained were free from the influence of interfering substances and correlated well with biological activities. Müller packed the adsorption column with three layers of alumina of different activities. The lowest layer consisted of the most active alumina which had a heat adsorption Q of 83.5 cal. The activities of the middle and upper layers varied with the problem. Vitamin A esters and vitamin A alcohol were separated easily.

Chromatography has been used by several investigators for the separation of free and esterified vitamin A. Awapara et al. (1946) used magnesia and alumina to estimate vitamin A in margarine. Quantitative separation of alcohol and ester forms of vitamin A of cod liver oil was done by solvent extraction and chromatography on activated alumina by Reed et al. (1944). They developed the column with ethylene dichloride.

Various early workers tried different adsorbents for estimation of vitamin A. A column of bone meal was used by Glover et al. (1947), Chilcote et al. (1949), and Dowler and Langhland (1952) for estimation of vitamin A in fish oils. Hjarde (1950) used dicalcium phosphate as adsorbent for separation of nonsaponifiable fraction of vitamin A in fish oil.

A method for the isolation of vitamin A in mixed feed was proposed by Cooley et al. (1945). They used sodium carbonate as the adsorbent. Cooley et al. (1949) improved the method by adopting an activated magnesia: Hyflo super cell mixture as adsorbent. In these experiments they isolated the ester form of vitamin A, the form generally present in mixed feeds of that period. In 1955, manufacturers of mixed feed started using three stabilized forms of vitamin A: (a) stabilized with gelatin, (b) stabilized with hydrogenated fat, (c) stabilized by microcrystalline wax. Vitamin A stabilized in gelatin was not soluble in petroleum solvents until the gelatin matrix had been hydrolyzed. Thus it became necessary to find a different method for isolation of vitamin A in mixed feed. Parrish (1957) hydrolyzed the feed with alcoholic KOH, and used magnesia for chromatographic separation of vitamin A. In 1958 and 1959 results were compared using magnesia and alumina (Parrish, 1958; 1959). "Woelm" alumina[1] was adopted for future

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[1] Woelm, Alumina. Neutral, activity grade 1. Alupharm Chemicals, New Orleans, La.

work because it is readily available and it is easy to prepare the partially deactivated adsorbent for use. The official method of A.O.A.C. (1960) for vitamin A in mixed feeds specifies "Woelm" alumina as an adsorbent for chromatography.

According to Rosenberg (1945), vitamin A is sensitive to oxidation. It is heat stable in an inert atmosphere and is stabilized in solution in oil. Vitamin A is destroyed by ultraviolet light and is optically inactive.

## MATERIALS AND METHODS

### Apparatus

The following apparatus was used in the study of the chromatographic separation of vitamin A on alumina according to the methods of analysis, A.O.A.C. (1960). All glass apparatus was used so that cork or rubber did not come in contact with the solutions.

Chromatographic Tubes. This apparatus consisted of a tube 18 x 200 mm, sealed to tube 5 x 100 mm. For experiments at different temperatures, the same size tube was used but was surrounded by a specially constructed water jacket, so that the column could be held at different temperatures by passing water at the desired temperature through the jacket.

Eluate Receiver. The eluate receiver used was the special equipment fraction collector, Corning no. 91200. The stem of the chromatographic tube was passed through a hole in a rubber stopper inserted at the top of the eluate receiver, and the eluate receiver was connected to an ordinary water aspirator for vacuum.

Saponification Apparatus. For saponification, the sample was placed in a 125-ml boiling flask. The flask was connected through a standard-

taper glass joint to a water condenser. Steam was used for heating.

Extraction Apparatus. The extraction apparatus used was an ordinary 250-ml glass-stoppered separatory funnel.

Photoelectric Colorimeter. The Evelyn Photoelectric colorimeter, with direct reading deflection type galvanometer, was used in these studies. A filter transmitting light at 620 mμ was used for the vitamin A determination and one transmitting light at 440 mμ for the carotene determination.

Carr-Price Reagent Dispenser. The Carr-Price reagent dispenser was a rapid delivering pipette having a delivery volume of 9 ml.

Evaporation Assembly. Vitamin A solutions for the Carr-Price test were evaporated directly in colorimetric tubes attached to a water aspirator, through an inert stopper and modified Y tube. Hot water at 60 to 65° served as the source of heat.

Ultraviolet Light. The ultraviolet light source used for observing the vitamin A bands was a Mineralight, model SL3660, Fisher Scientific Co., Cat. no. 11-984-1. Two other ultraviolet light sources were used to study the effects of radiation on recoveries of vitamin A and carotene during chromatography. One was a germicidal light (8W, General Electric), and the other was a Black Raymaster, (8W, Geo. W. Gates and Co. Franklin Sq, L.I.N.Y.).

Projector Floodlight. To study the effect of bright radiation by ordinary light on recovery of vitamin A during chromatography, a "Sylvania" 150 W, 120 V Projector Floodlight was used.

## Reagents

Adsorbents. Three types of "Woelm" (Eschwege) alumina were used as

adsorbents: (a) neutral, activity grade 1; (b) basic, (cation-tropic), activity grade 1; (c) acidic (anion-tropic), activity grade 1. Five percent water was added to each alumina by placing the measured quantity of water in a small bottle and distributing it over the walls. Alumina was added and mixed by shaking the bottle until no lumps were visible. The alumina was allowed to cool at least two hours before use. It was stored in a tightly closed bottle. Precautions were taken not to expose the original or prepared alumina to air, in order to control the moisture content. The resulting alumina had an activity grade of between 2 to 3 on the Brockmann and Schodder (1941) Scale.

Chloroform. Reagent grade chloroform (A.C.S.) was used.

Hexane. Skellysolve B (commercial hexane) was used. It was redistilled from an all-glass apparatus, using the 64 to 68° fraction. Care was taken to use hexane which was free of alcohols, esters and moisture.

Alcohol. Ninety-five percent, reagent grade (A.C.S.), aldehyde-free (by Schiff's test) ethanol was used.

Acetone-in-Hexane Solution. Four percent and 15 percent acetone (reagent grade), A.C.S., in hexane was used.

Solution for Removing Antimony Trichloride from Tubes. Tubes were placed in 10 percent Rochelle Salt solution to which detergent was added. Then tubes were washed thoroughly in hot detergent solution.

Potassium Hydroxide Solution. Fifty percent reagent grade potassium hydroxide in water (W/V) was used for saponification.

Antimony Trichloride. (Carr-Price) Reagent. About 900 ml chloroform was added to 200 gm reagent grade, A.C.S., antimony trichloride. It was warmed and shaken. It was kept warm for about 24 hours with occasional

shaking to allow the solid to dissolve. The volume was made to one liter by addition of chloroform. Thirty milliliters of acetic anhydride then was added. After shaking, the solution was allowed to stand for sometime so that any sediment might settle. The solution was filtered and kept in a tightly stoppered brown bottle.

Vitamin A Solution. A solution of crystalline trans vitamin A acetate in cottonseed oil, encapsulated in gelatin (U.S.P. Vitamin A Reference Solution) was used. Each capsule contained 250 mg of a solution prepared to contain 30 mg vitamin A per gm of oil.

Carotene. Twenty-four percent β-carotene, semi-solid suspension in vegetable oil, (Hoffmann-La Roche Inc., New Jersey) was dissolved in hexane and used to make the vitamin A-carotene mixture.

## Preparation of Adsorption Column

In this study, the method followed for preparation of adsorption column was essentially as recommended by the A.O.A.C., (1960). A small piece of cotton was placed at the bottom of the chromatographic tube. The adsorbent mixture was added gradually to the tube and was set properly by tapping the alumina in the column with a blunt rod. Thus a 7-cm column was made, over which a 0.5-cm layer of anhydrous sodium sulphate was added. During packing, the column was kept under vacuum, (ordinary water aspirator was used for vacuum).

## Determination

Saponification. The form of vitamin A used in feeds as well as some of the carotenoids occur in the forms of esters, so that it is necessary

to saponify before separation of them on the column. In case of gelatin stabilized vitamin A, it also is necessary to digest the gelatin to free vitamin A. In the natural state carotene and vitamin A are present combined with tissues. Saponification helps to decompose these tissues, freeing carotene and vitamin A. Carotenoids sometimes are associated with large amount of lipid materials. By saponification these lipids are hydrolyzed and can be removed. Saponification is done by digesting the sample with 10 percent alcoholic potassium hydroxide solution on a water bath for one-half hour, using a reflux condenser. The saponification changes the ester forms of carotenoids and vitamin A to the alcoholic forms. The nonsaponifiable fraction of carotenoids and vitamin A is extracted by hexane.

When a mixture of vitamin A ester and carotene is passed through the column, first vitamin A is eluted and then carotene. Sometimes a mixture of these two substances is difficult to separate. After saponification this difficulty is overcome. In this case carotene is eluted first and then vitamin A alcohol. Thus satisfactory separation is possible.

In this study saponification and extraction were done by an adaptation of the official methods of analysis, A.O.A.C., (1960). One ampule of U.S.P. Vitamin A Acetate Reference Solution was used. The narrow end of the ampule was cut by a scissors, and the solution was squeezed out completely into a 125-ml saponification flask. One gram of cottonseed oil, 10 ml alcohol, and 10 ml of 50 percent KOH solution were added. The solutions were mixed thoroughly and refluxed for 30 minutes at the rate of two drops per second. During digestion the flask was shaken occasionally. The flask was cooled to room temperature under running water, and 25 ml of water was added. The

mixture was extracted twice with 50 ml hexane, in two different separatory funnels.

Hexane extracts were washed with water by pouring 100 ml cold water into the separatory funnels. After the addition of water, the solutions in the separatory funnels were allowed to settle a few minutes. When the layers had separated, the water was drained, retaining any small amount of emulsion in the hexane. After three more washings the extracts in the two funnels were combined and washed again with water. When there was difficulty with emulsions, a few ml of alcohol was added, and after the lower layer was removed, the extract was washed again with water. The solution was allowed to stand about five minutes and then the few drops of water left were drained as completely as possible. The solution was poured carefully from the top of the separatory funnel through a small piece of cotton into 100-ml volumetric flask. Separatory funnel and cotton were rinsed with small portions of hexane, which was added to the solution in the volumetric flask, and the solution was made to 100 ml with hexane.

Chromatography. An aliquot containing at least 30 mmg vitamin A is preferred for chromatography, but there were slight variations in the quantities used. In no case was more than 25 ml solution chromatographed. The column was packed as mentioned before, and washed with 20 ml hexane. The vitamin A-carotene solution was added on the top of the column before the top of the column became dry. Throughout the chromatography the flow rate was adjusted to one or two drops per second, using a slight vacuum from a water aspirator to adjust the flow rate. After adsorption, the carotene was eluted by about 20 ml of 4 percent acetone in hexane and location of the vitamin A band was checked using the ultraviolet light. After

carotene was eluted, vitamin A was eluted with about 25 ml of 15 percent acetone in hexane. The column was washed with 15 percent acetone until there was no fluorescence due to vitamin A in the elute, as observed by use of the ultraviolet light. The eluates of vitamin A and of carotene were made to measured volumes and analyzed.

Colorimetry. The transmittancies were read on the Evelyn photometer. Ten milliliters of the carotene solution (from chromatographic separation) was placed in a colorimetric tube and the yellow color was read at 440 mμ as carotene. Ten milliliter of vitamin A solution from chromatographic separation was taken and yellow color read at 440 mμ. The 10 ml of vitamin A solution then was evaporated to dryness (in the colorimetric tube itself) under vacuum in a hot (60 to 65°) water bath. The residue was dissolved in one ml chloroform. The 620 mμ filter was inserted in the colorimeter and the galvanometer was set at 100 percent transmission, using one ml chloroform and 9 ml antimony trichloride reagent. The tube containing vitamin A in chloroform was placed in the instrument and antimony trichloride was added rapidly; the maximum reading of the galvanometer was taken within 5 seconds. If done under proper conditions, the solution should be blue, without turbidity, and the blue color of the solution should fade rapidly.

Vitamin A and carotene absorbancies were calculated by using the formula: absorbancy = 2 - log T, (T = transmittance). The values for absorbancies were obtained directly from tables supplied for use with the Evelyn photometer. Absorbancy of carotene, when multiplied by 2.86[1], gave

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[1]The values 2.86, 0.2 and 12.6 were obtained in this laboratory by calibration of the Evelyn photometer, using the standard solutions of vitamin A and β-carotene.

the carotene content in mmg (micrograms) per ml.

Absorbancy of vitamin A due to the Carr-Price reaction products was corrected for that due to accompanying yellow pigments in the following manner: The galvanometer was read for the yellow color of the vitamin A solution at 440 mμ and absorbancy was calculated. Absorbancy was multiplied by 0.2[1], and this value was subtracted from the absorbancy obtained at 620 mμ. The corrected absorbancy was multiplied by 12.6[1] to obtain the vitamin A content in mmg per 10 ml.

## RESULTS AND DISCUSSION

### Effect of Temperature

In the first experiment, the effects of different temperatures on the separation and recovery of vitamin A and carotene were studied to determine whether this might be a factor effecting the results obtained by the A.O.A.C. method.

In this experiment, chromatographic separation and recovery of vitamin A and β-carotene from a mixture of solutions of them was studied at two different concentrations (hereafter named $C_1$ and $C_2$), at different temperatures. Vitamin A and carotene stock solutions were prepared by saponification and extraction of one capsule of Vitamin A Reference Solution (U.S.P.) and addition of carotene, as mentioned under Materials and Methods. The $C_1$ solution was prepared by diluting 10 ml of the stock solution to 250 ml. Ten-milliliter portions of this $C_1$ solution were chromatographed at

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[1]The values 2.86, 0.2 and 12.6 were obtained in this laboratory by calibration of the Evelyn photometer, using the standard solutions of vitamin A and β-carotene.

five different temperatures, $0^{\circ}$, $13^{\circ}$ (tap water), $23^{\circ}$ (room temperature), $38^{\circ}$ and $48^{\circ}$, respectively. These temperatures were obtained by passing water through the outer jacket of the special chromatographic tube. The temperature of the water in the reservoir from which water was circulating through the outer jacket was recorded by a thermometer. The temperature was kept constant (variation of approximately $\pm 1^{\circ}$) by passing steam slowly into the water or adding ice as required. After elution, the final volume of the solution containing carotene was made to 25 ml, and the solution containing vitamin A, to 50 ml.

The $C_2$ solution was made by diluting 1 ml of the stock solution to 100 ml. Twenty-five milliliter of this $C_2$ solution was chromatographed as with solution $C_1$.

It was observed that at lower temperatures carotene was eluted slowly, whereas at higher temperatures the carotene was eluted more quickly. For chromatography, $48^{\circ}$ is not a reasonable temperature, as the solvent almost boils at this temperature. So, after a few trials at $48^{\circ}$, the study was limited to the following four temperatures: $0^{\circ}$, $13^{\circ}$, $23^{\circ}$, and $38^{\circ}$, and results at $48^{\circ}$ were not included in the statistical analysis. Eight observations were taken on each of the solutions $C_1$ and $C_2$, at each of the four temperatures. Results on vitamin A and carotene determinations at $23^{\circ}$ were taken as standards and percent deviations from the standards at the other temperatures, $0^{\circ}$, $13^{\circ}$ and $38^{\circ}$, were calculated. (Table 1). The analysis of variance (Snedecor, 1960)[1] and the F test show that there was a significant

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[1]See appendix for sample calculation.

difference in results on vitamin A (P = .01) due to the effect of temperature. The L.S.D.[1] was calculated and it was found that the loss of vitamin A at 38° was significant.

The analysis of variance for carotene was calculated. The F test indicates a significant difference in carotene (P = .01) due to the effect of temperature. The L.S.D. shows that the loss of carotene at 38° was significant.

The loss of vitamin A and carotene at 38° may be due to destruction in the process of chromatography or, possibly, partly due to isomerization. Rosenberg (1945) describes vitamin A as heat stable in an inert atmosphere. The present study shows that when the analysis was carried out at 38° there was a significant loss of vitamin A and carotene. Deuel (1951) mentions that cis-trans isomerization of carotene and vitamin A can occur due to the effect of heat. So possibly part of the loss may be due to isomerization.

## Effect of Different Varieties of Alumina

Chromatographic separation and recovery of vitamin A and carotene was done on the following three types of alumina: (1) Neutral alumina, activity grade 1, pH 6.8-7.2. The pH was determined on a slurry made of 20 gm alumina and 25 ml water. The pH was 6.8 after the slurry was allowed to settle, whereas the immediate reading was pH 7.2. (2) Basic alumina (cation-tropic), activity grade 1, had pH 10.2 (pH determined as above) when determined after the solid was allowed to settle, but pH 11.8 when determined immediately. (3) Acidic alumina (anion-tropic), activity grade 1, had pH 4.2, both immediately after the slurry was stirred and after the solid was allowed to settle.

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[1] Least significance difference.

Table 1. Chromatographic separation and recovery of carotene and vitamin A at different temperatures[a].

| | Temperatures | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0°C | | 13°C | | 23°C | | 38°C | | 48°C[b] | |
| | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ |
| Carotene, mmg/ml | .7437 | .8690 | .7384 | .8594 | .7282 | .8552 | .7150 | .8347 | .75 | .81 |
| % Deviation | 2.1271 | 1.6130 | 1.4007 | .4901 | S | S | -1.8260 | -2.397 | -3.85 | -3.57 |
| Vitamin A, mmg/10 ml | 4.2383 | 2.3122 | 4.2110 | 2.2808 | 4.2029 | 2.2869 | 4.0489 | 2.2077 | 3.93 | 2.04 |
| % Deviation | .8422 | 1.1111 | .1927 | -.2670 | S | S | -3.6641 | -3.4588 | -2.48 | -7.27 |
| No. of trials | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 4 | 3 |

Analysis of variance for carotene:

| Sources | d.f. | S.S. | F. |
|---|---|---|---|
| Temperature | 2 | 17.3682 | 377.57** |
| Concentration | 1 | .6638 | |
| Residual | 2 | .0460 | |

L.S.D. Carotene = 0.6523

Analysis of variance for vitamin A:

| Sources | d.f. | S.S. | M.S. | F. |
|---|---|---|---|---|
| Temperature | 2 | 22.6952 | 11.3476 | 139.58** |
| Concentration | 1 | 0 | 0 | |
| Residual | 2 | 0.1629 | 0.0814 | |

L.S.D. Vitamin A = 1.2276

a. On this table and tables 2 to 7 which follow, these symbols are used:

$C_1$, $C_2$ Two different concentrations of test solutions.
S. Standard to which other values are compared.
d.f. Degrees of freedom.
S.S. Sum of squares.
M.S. Mean square.
F. Variance ratio. * = Significant. ** = Highly significant.
L.S.D. Least significance difference.

b. Results of 48° omitted from analysis of variance.

The stock solution of vitamin A and carotene was prepared as in experiment 1. For chromatography, 3 ml of stock solution was diluted to 200 ml; of this 25 ml was chromatographed on neutral, basic and acidic aluminas, each at three different temperatures: $0^{\circ}$, $23^{\circ}$, and $38^{\circ}$. Final volumes of solutions of carotene and vitamin A were made to 50 ml. Ten milliliter was analyzed.

Eight observations were taken using each kind of alumina at each temperature. Values for carotene and vitamin A obtained at $23^{\circ}$ using neutral alumina were taken as standard. The percent deviations of vitamin A and carotene from the standards at $0^{\circ}$, $23^{\circ}$ and $38^{\circ}$ obtained using different kinds of alumina are recorded in Table 2. The analysis of variance for vitamin A was calculated and F test indicates that there was a significant difference due to the effect of kind of alumina ($P = .05$). The F test for temperature also shows that there was a significant difference in vitamin A values due to the effect of temperature ($P = .05$). The L.S.D. for different varieties of alumina shows that the loss of vitamin A on acidic alumina was significant ($P = .05$). The L.S.D. for temperature also reveals a significant loss of vitamin A at $38^{\circ}$.

The analysis of variance for carotene was calculated, and the F test shows that there was a significant difference on carotene due to different varieties of alumina ($P = .01$). The F test reveals a significant difference in results on carotene due to the effect of temperature. The L.S.D. for different varieties of alumina was calculated and it was found that the loss of carotene on acidic alumina was significant. The L.S.D. for different temperatures also was calculated and it was found that the loss of carotene at $38^{\circ}$ was significant. The loss of vitamin A and carotene due to effect of

temperature was observed also in the previous experiment.

It is mentioned by Moore (1957) that both vitamin A and carotene are stable in alkali in the absence of oxygen. It is found in the present study that there was no significant difference in effect of basic alumina as compared to neutral alumina on the chromatographic separation of carotene and vitamin A.

It is mentioned by Deuel (1951) that both isomerization and/or destruction of vitamin A and carotenoids may take place by treatment with acids. The same thing also is mentioned by Sebrell and Harris (1954). In the present study it was found that there was a significant loss of vitamin A and of carotene when acidic alumina was used in chromatography. It is possible that the loss may be due to destruction of vitamin A and carotene; some loss may be due to isomerization also.

### Effect of Nonsaponifiable Fraction of Lipid

In the third experiment, the effects of addition of nonsaponifiable fraction of lipid on the separation and recovery of vitamin A and carotene were studied to determine whether this might be a factor influencing the results obtained by the A.O.A.C. method.

In this experiment the chromatographic separation of the vitamin and β-carotene mixture was studied using a vitamin A solution saponified and extracted without the addition of oil. Vitamin A was saponified and extracted as in experiment 1, except that cottonseed oil was not added. Then, 1 ml of cottonseed oil was saponified separately under the same conditions as for vitamin A and extracted with 50 ml of hexane.

One hundred ml of vitamin A stock solution was prepared with addition

Table 2. Chromatographic separation and recovery of vitamin A and carotene using different varieties of alumina.

| Alumina used | Component | Temperatures | | |
|---|---|---|---|---|
| | | 0°C | 23°C | 38°C |
| Neutral | Carotene, mmg/ml | .6292 | .6222 | .5994 |
| % Deviation | | 1.125 | S | -3.6655 |
| Acidic | Carotene, mmg/ml | .5935 | .5715 | .5524 |
| % Deviation | | -4.612 | -8.1485 | -11.2182 |
| Basic | Carotene, mmg/ml | .6221 | .6248 | .5923 |
| % Deviation | | -.0160 | .4178 | -4.8055 |
| Neutral | Vitamin, mmg/10 ml | 3.6550 | 3.6268 | 3.5553 |
| % Deviation | | .7775 | S | -1.9714 |
| Acidic | Vitamin, mmg/10 ml | 3.5665 | 3.4520 | 3.3178 |
| % Deviation | | -1.6620 | -4.8190 | -8.5199 |
| Basic | Vitamin, mmg/10 ml | 3.6545 | 3.6019 | 3.5256 |
| % Deviation | | .7656 | -.6865 | -2.7900 |
| Number of trials with each alumina. | | 8 | 8 | 8 |

Analysis of variance for vitamin A:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Alumina | 2 | 38.2199 | 19.1099 | 15.99* |
| Temperature | 2 | 29.1920 | 14.5960 | 12.21* |
| Residual | 4 | 4.7804 | 1.1951 | |

Analysis of variance for carotene:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Alumina | 2 | 94.0276 | 47.0138 | 44.47** |
| Temperature | 2 | 46.9825 | 23.4912 | 22.22** |
| Residual | 4 | 4.2284 | 1.0571 | |

L.S.D. Carotene = 2.3302 L.S.D. Vitamin A = 2.4790

of carotene. Ten ml of this solution was diluted to 250 ml. Ten ml of the diluted solution was taken and 1 ml of hexane was added to it; to another 10 ml of the same solution 1 ml of unsaponifiable fraction extracted from oil was added. Each of the above two solutions was chromatographed through alumina columns at $0^{\circ}$, $23^{\circ}$, and $38^{\circ}$. Final volume of carotene solution was made to 25 ml and that of vitamin A to 50 ml. Results were compared on the solutions subjected to the different treatments.

Table 3 shows the percent deviations of vitamin A and carotene at $0^{\circ}$ and $38^{\circ}$ from the standard value obtained at $23^{\circ}$ when unsaponifiable fraction of oil was used. The analysis of variance shows that there was no significant loss of vitamin A or carotene when the nonsaponifiable fraction of lipid was omitted ($P = .05$). Wilkie (1952) states that there may be loss of vitamin A in the saponification of synthetic vitamin A preparations due to lack of antioxidants. This loss may be prevented by addition of cottonseed oil or pyrogallic acid or both. In the present study, in contrast to the findings of Wilkie (1952), there was no significant loss of either vitamin A or carotene when nonsaponifiable fraction of lipid was omitted.

The analysis of variance for carotene shows that there was a significant difference on carotene due to the effect of temperature ($P = .01$). After calculating the L.S.D. it was found that the loss of carotene at $38^{\circ}$ was significant, in agreement with findings on carotene in experiment 1.

## Effect of Radiation

In this experiment the chromatographic separation of vitamin A and β-carotene mixtures were studied, under illumination from normal laboratory ceiling lights, from a photoflood lamp, and from two types of ultraviolet lights.

Table 3. Chromatographic separation and recovery of vitamin A and carotene with and without addition of nonsaponifiable fraction of lipid.

| | With addition of nonsaponifiable fraction of lipid | | | Without addition of nonsaponifiable fraction of lipid | | |
|---|---|---|---|---|---|---|
| | 0°C | 23°C | 38°C | 0°C | 23°C | 38°C |
| Vitamin A, mmg/10 ml | 4.0160 | 4.0241 | 3.9506 | 3.9734 | 4.0228 | 3.9124 |
| % Deviation | -0.2003 | S | -1.8030 | -1.260 | -0.0320 | -2.7750 |
| Carotene, mmg/ml | .7146 | .7100 | .6985 | .7118 | .7099 | .6964 |
| % Deviation | .6478 | S | -1.6197 | .2535 | -0.0140 | -1.9154 |
| Number of trials | 8 | 8 | 8 | 8 | 8 | 8 |

Analysis of variance for vitamin A:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Lipids | 1 | .7098 | .7098 | 4.36 |
| Temperature | 2 | 5.4044 | 2.7022 | 16.59 |
| Residual | 2 | .3245 | .1628 | |

Analysis of variance for carotene:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Lipids | 1 | .0826 | .0826 | 4.2359 |
| Temperatures | 2 | 5.4863 | 2.7432 | 140.6769** |
| Residual | 2 | .0389 | .0195 | |

L.S.D. Carotene = 4.3030

Vitamin A stock solution was prepared as in experiment 3. One ml of cottonseed oil was saponified and extracted under the same conditions as for vitamin A and the volume was made to 50 ml. From the stock solution of vitamin A-carotene mixture, two solutions, $C_1$ and $C_2$ were made. For solution $C_1$, 4 ml of stock solution was diluted to 100 ml, for solution $C_2$, 2 ml was diluted to 100 ml.

The first series of trials were done using a vitamin A and carotene mixture with addition of nonsaponifiable fraction of cottonseed oil. Ten ml of $C_1$ and of $C_2$ were taken, 1 ml of hexane added to each, and each was

chromatographed under the following conditions: (a) normal laboratory ceiling lights; (b) "Sylvania", 150 W, 120 V, Projector flood (at a distance of one foot); (c) ultraviolet light, 8 Watt, Germicidal (at a distance of one foot); and (d) ultraviolet light, Black Raymaster, Type B, 8 Watt, Radiation peak 3600 $A^{O}$ (at a distance of one foot).

The second series of trials were made as above, except for the addition of 1 ml of nonsaponifiable fraction of cottonseed oil. Carotene and vitamin A were eluted from the column using 25 ml of 4 percent, and 25 ml of 15 percent acetone in hexane solutions, respectively. Final volumes of both the vitamin A and carotene solutions were made to 50 ml, and 10 ml of these extracts were used for analysis.

The effects of normal laboratory ceiling light and strong light (photoflood) on the chromatographic separation and recovery of carotene and vitamin A are shown in Table 4 for each of the solutions, $C_1$ and $C_2$, with and without nonsaponifiable fraction of oil. Results on the $C_1$ and $C_2$ solutions with nonsaponifiable fraction of oil under ordinary laboratory light were taken as standards.

The analysis of variance shows that there was a significant difference in results on vitamin A due to the effect of light ($P = .05$). The L.S.D. indicates that bright light was responsible for a significant loss of vitamin A ($P = .01$). It is apparent that there was no significant loss of vitamin A due to the presence or absence of nonsaponifiable fraction of oil.

The analysis of variance shows that there was a significant difference in results on carotene due to the effect of light ($P = .05$). The L.S.D. reveals that bright light was responsible for effecting a significant loss of carotene ($P = .05$).

Table 4. Chromatographic separation and recovery of carotene and vitamin A under normal laboratory ceiling light and strong incandescent light[a].

| | With nonsaponifiable fraction of oil | | | | Without nonsaponifiable fraction of oil | | | |
|---|---|---|---|---|---|---|---|---|
| | Ordinary light | | Strong light | | Ordinary light | | Strong light | |
| | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ |
| Carotene, mmg/ml | .7192 | .4357 | .7094 | .4272 | .7172 | .4252 | .7132 | .4215 |
| % Deviation | S | S | -1.3626 | -1.9500 | -0.2363 | -2.4099 | -0.8342 | -3.2591 |
| Vitamin A, mmg/10 ml | 4.8346 | 2.6177 | 4.6966 | 2.5142 | 4.7480 | 2.5951 | 4.6296 | 2.5085 |
| % Deviation | S | S | -2.8544 | -3.9538 | -1.7912 | -0.8633 | -4.2402 | -4.1716 |
| No. of trials | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

Analysis of variance for carotene:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Light | 1 | 2.8323 | 2.8323 | 15.8317* |
| Solution | 3 | 6.8413 | 2.2804 | 12.7468 |
| Residual | 3 | .5366 | .1789 | |

L.S.D. Carotene = 0.9517

Analysis of variance for vitamin A:

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Light | 1 | 19.7365 | 19.7365 | 94.7504** |
| Solution | 3 | 2.8169 | .9389 | 4.5074 |
| Residual | 3 | .6248 | .2083 | |

L.S.D. Vitamin A = 1.0268

[a]All studies carried out at room temperature (23° to 25°).

The loss of vitamin A and carotene by the effect of light may be due to destruction or some irreversible chemical reactions; some loss may be due to isomerization also.

Table 5 shows percent deviations of results on carotene and vitamin A when chromatography was performed on both $C_1$ and $C_2$ solutions under irradiation from untraviolet lights, with and without the addition of nonsaponifiable fraction of oil. Values for vitamin A and carotene obtained with nonsaponifiable fraction of oil under ordinary ceiling light are taken as standards.

Regarding vitamin A, the analysis of variance shows that there were no significant differences in vitamin A of both $C_1$ and $C_2$ solutions due to the effect of ultraviolet light. There were also no significant differences in vitamin A of $C_1$ and $C_2$ solutions due to the effect of nonsaponifiable fraction of lipid.

In case of carotene, the analysis of variance shows that there were no significant differences in carotene of both $C_1$ and $C_2$ solutions due to the effect of ultraviolet light. There was no significant differences in carotene due to omission of nonsaponifiable fraction of lipid.

The present study indicates that there was no significant effect of ultraviolet light on the chromatographic separation of vitamin A and carotene, which is in contrast to the findings of some other workers. Neal and Luckmann (1944) and Little (1944) mentioned that ultraviolet light causes destruction of vitamin A and carotene. A probable reason for the nonsignificant losses of vitamin A and carotene in the present study may be the fact that the ultraviolet lights used were not powerful enough to cause destruction or that most of the radiations were absorbed by the chromatographic

Table 5. Chromatographic separation and recovery of carotene and vitamin A under ultraviolet and ordinary laboratory ceiling light[a].

| Light Source | Without addition of nonsaponifiable fraction of oil | | | | With addition of nonsaponifiable fraction of oil | | | |
|---|---|---|---|---|---|---|---|---|
| | Vitamin A, mmg/10 ml | | Carotene, mmg/ml | | Vitamin A, mmg/10 ml | | Carotene, mmg/ml | |
| | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ | $C_1$ | $C_2$ |
| Ordinary light | 4.4081 | 2.0898 | .7185 | .3470 | 4.4614 | 2.0836 | .7140 | .3497 |
| % Deviation | -1.1946 | .2966 | .6302 | -0.7720 | S | S | S | S |
| Black Raymaster | 4.3811 | 2.0268 | .7027 | .3417 | 4.3892 | 2.0736 | .7027 | .3503 |
| % Deviation | -1.7998 | -2.7260 | -1.5826 | -0.2287 | -1.6183 | -0.4799 | -1.5826 | .1715 |
| Germicidal | 4.3273 | 2.0385 | .6948 | .3414 | 4.3780 | 2.0754 | .7071 | .3473 |
| % Deviation | -3.0005 | -2.1645 | -2.6890 | -2.3734 | -1.8693 | -0.3935 | -.9663 | -.6863 |
| No. of trials each lamp and solution | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

Analysis of variance:

| | Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|---|
| For vitamin A: $C_1$ solution | Light | 2 | 3.4264 | 1.7132 | 10.641 |
| | Lipids | 1 | 1.0478 | .5239 | 3.254 |
| | Residual | 2 | .322 | .161 | |
| For vitamin A: $C_2$ solution | Light | 2 | 3.4677 | 1.7389 | 1.897 |
| | Lipids | 1 | 2.3070 | 2.3070 | 2.517 |
| | Residual | 2 | 1.8327 | .9164 | |
| For carotene: $C_1$ solution | Light | 2 | 5.5018 | 2.7509 | 3.708 |
| | Lipids | 1 | .1989 | .1989 | .268 |
| | Residual | 2 | 1.4836 | .7418 | |
| For carotene: $C_2$ solution | Light | 2 | 2.4599 | 1.2299 | 5.608 |
| | Lipids | 1 | 1.3626 | 1.3626 | 6.213 |
| | Residual | 2 | .4386 | .2193 | |

[a]All studies carried out at room temperature (23° to 25°).

glass tube, which was made of pyrex glass.

## Effect of Minerals

In this experiment the chromatographic separation of vitamin A and carotene was studied on alumina to which trace amounts of copper and iron salts were added. A mixture of vitamin A and carotene stock solution was prepared as in previous experiments. Two different concentrations, $C_1$ and $C_2$, were prepared by diluting 4 ml and 2 ml of stock solution to 100 ml with hexane.

Aluminas containing trace quantities of minerals were prepared as follows: Mineral salts were weighed and dissolved in a measured volume of water such that 2.5 ml of salt solution when added to 50 gm of alumina provided the required concentration of minerals, and also the 5 percent moisture content of chromatographic alumina. Salts used were $CuSO_4 \cdot 5H_2O$ and $FeSO_4 \cdot 7H_2O$. Aluminas containing .001 percent copper, .001 percent iron, and .01 percent iron, were prepared. One ml hexane or one 1 ml of nonsaponifiable fraction of oil was added to 10 ml of vitamin A solution. These mixtures were chromatographed on each of the three columns. Carotene and vitamin A were eluted by 4 percent and 15 percent acetone in hexane, respectively.

Table 6 shows the effect of the above mentioned minerals on the chromatographic separation and recovery of carotene and vitamin A. The values obtained for vitamin A and carotene in both $C_1$ and $C_2$ solutions with addition of nonsaponifiable fraction of oil are taken as standards. There was a significant difference of vitamin A ($P = .05$) in the $C_2$ solution due to the effect of minerals. The L.S.D. shows a significant loss of vitamin A in the $C_2$ solution when chromatographed on alumina containing .01 percent iron. There was

Table 6. Chromatographic separation and recovery of carotene and vitamin A on alumina containing trace quantities of mineral[a].

| | With addition of nonsaponifiable fraction of oil | | | | Without addition of nonsaponifiable fraction of oil | | | |
|---|---|---|---|---|---|---|---|---|
| | $C_1$ | | $C_2$ | | $C_1$ | | $C_2$ | |
| | [1]Car., mmg/ml | Vit. A, mmg/10 ml | Car., mmg/ml | Vit. A, mmg/10 ml | Car., mmg/ml | Vit. A, mmg/10 ml | Car., mmg/ml | Vit. A, mmg/10 ml |
| Pure alumina | .7095 | 4.5479 | .3642 | 2.3224 | .7178 | 4.5856 | .3555 | 2.2928 |
| % Deviation | S | S | S | S | 1.1698 | .8285 | -2.3887 | -1.2745 |
| Alumina with .001% Cu | .7137 | 4.4961 | .3518 | 2.2944 | .7046 | 4.4249 | .3472 | 2.2909 |
| % Deviation | .5919 | -0.6992 | -3.4077 | -1.2056 | -0.6906 | -2.7045 | -4.6677 | -1.3563 |
| Alumina with .001% Fe | .7184 | 4.3415 | .3631 | 2.2850 | .7138 | 4.3677 | .3571 | 2.2908 |
| % Deviation | 1.2544 | -4.5383 | -0.3020 | -1.6104 | .6060 | -3.9622 | -1.9494 | -1.3606 |
| Alumina with .01% Fe | .6972 | 4.2808 | .3469 | 2.2447 | .6976 | 4.3022 | .3468 | 2.2176 |
| % Deviation | -1.7336 | -5.8730 | -4.7501 | -3.3456 | -1.6772 | -5.4024 | -4.7775 | -4.5125 |
| No. of trials | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

Analysis of variance:

| | Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|---|
| For vitamin A: $C_1$ solution | Minerals | 3 | 43.3885 | 14.4628 | 1.6505 |
| | Lipids | 1 | .0021 | .0021 | .0002 |
| | Residual | 3 | 26.2870 | 8.7623 | |
| For vitamin A: $C_2$ solution | Minerals | 3 | 12.4975 | 4.1658 | 14.7097* |
| | Lipids | 1 | .6858 | .6858 | 2.4216 |
| | Residual | 3 | .8498 | .2832 | |
| For carotene: $C_1$ solution | Minerals | 3 | 8.2077 | 2.7359 | 4.9554 |
| | Lipids | 1 | .0621 | .0621 | .1124 |
| | Residual | 3 | 1.6563 | .5521 | |
| For carotene: $C_2$ solution | Minerals | 3 | 21.5291 | 7.1430 | 14.6643* |
| | Lipids | 1 | 3.5465 | 3.5465 | 7.2808 |
| | Residual | 3 | 1.4613 | .4871 | |

L.S.D. Carotene = 2.2207 L.S.D. Vitamin A = 1.6935

[1]Carotene.

[a]All studies carried out at room temperature (23° to 25°).

no significant difference in vitamin A of the $C_2$ solution when nonsaponifiable fraction of oil was omitted.

In the cases of both carotene and vitamin A in the $C_1$ solution, the analysis of variance shows that there was no significant loss due to the effect of minerals. There also was no significant loss of carotene and vitamin A when nonsaponifiable fraction of oil is omitted.

Sebrell and Harris (1954) stated that metal ions, especially copper and cobalt, accelerate the oxidative destruction of vitamin A. Losses of vitamin A and carotene found in the present study may be due to this oxidative destruction. In this study metal ions caused more destruction of vitamin A and carotene in the dilute solutions than in the more concentrated solutions.

## Effect of Air

Chromatographic separation of a vitamin A and β-carotene solution was studied on columns treated as follows: (a) A column preconditioned by drawing air from laboratory through the column for 15 minutes or 30 minutes. (b) A column preconditioned by drawing dry air through the column for 15 minutes or 30 minutes. (Dry air was obtained by passing air through a calcium chloride tube). (c) An ordinary alumina column as used in previous work.

A vitamin A and carotene stock solution was prepared as in experiment 1, and 4 ml of this solution was diluted to 100 ml by hexane. Ten ml of the diluted vitamin A and carotene solution was chromatographed and eluted from each of the above columns. In all these experiments carotene and vitamin A were eluted by 4 percent and 15 percent acetone in hexane solutions,

respectively. Final volume of carotene eluate was made to 25 ml, and vitamin A eluate to 50 ml.

In the course of elution of vitamin A through ordinary and aerated alumina columns, as mentioned above, the following observations were made: (1) Twenty-five ml of 15 percent acetone in hexane was required for elution of vitamin A from ordinary alumina. (2) Twenty ml of the 15 percent acetone-hexane solution was sufficient for elution of vitamin A from alumina aerated with normal laboratory air. (3) Fifty to 55 ml of 15 percent acetone-hexane was required for elution of vitamin A from alumina aerated with dry air for 15 minutes. (4) Sixty to 65 ml of the 15 percent acetone-hexane solution was required for the elution of vitamin A from alumina aerated with dry air for one-half hour. These observations indicated that when atmospheric air was passed through the column, the activity of alumina was decreased, and vitamin A was eluted more quickly, requiring a lesser amount of solvent. However, when the dry air was drawn through the column, the activity of alumina was increased and vitamin A was held tightly on the column; thus more solvent was required.

It is known that the activity of alumina decreases as the moisture content increases. The moisture content of the alumina through which atmospheric air was drawn for half an hour was found to be 10.6 percent. Ordinary alumina two hours after the addition of 5 percent water was found to contain 3.4 percent moisture, whereas alumina through which dry air was drawn for one half hour, contained only 1.9 percent moisture. The moisture content was determined by heating alumina samples overnight in a muffle furnace (approximately 650°), cooling in desiccator, and weighing the samples. The loss in weight was taken as the moisture content of the alumina.

The percent deviations in results on vitamin A and carotene due to the effect of aeration with dry air and atmospheric air are shown in Table 7. The analysis of variance for vitamin A shows that there was a significant difference in results on vitamin A due to the effect of air. The L.S.D. reveals that dry air was responsible for the significant loss of carotene.

The loss of vitamin A and carotene due to the effect of dry air may result from oxidative destruction. Rosenberg (1945) mentions that vitamin A and carotene are sensitive to oxidation and autooxidize readily. Moore (1957) also states that both vitamin A and carotene are destroyed by oxidation. Alumina may act as a catalytic agent causing destruction. Some of the vitamin A, however, may be merely retained on the column, causing apparent loss.

Table 7. Chromatographic separation and recovery of vitamin A and carotene using alumina preconditioned with air[a].

| | Alumina | | | | |
|---|---|---|---|---|---|
| | Ordinary | Aerated by laboratory air | | Aerated by dry air | |
| | | 15 minutes | 30 minutes | 15 minutes | 30 minutes |
| Carotene, mmg/ml | .8509 | .8411 | .8396 | .8473 | .8461 |
| % Deviation | S | -1.1517 | -1.3280 | -0.4230 | -0.5641 |
| Vitamin A, mmg/10 ml | 3.8628 | 3.7359 | 3.7225 | 3.4700 | 3.4271 |
| % Deviation | S | -3.2851 | -3.6320 | -10.1687 | -11.2793 |
| No. of trials | 8 | 8 | 8 | 8 | 8 |

Analysis of variance:

| | Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|---|
| For vitamin A | Aeration | 1 | 52.7868 | 52.7868 | 362.20* |
| | Time | 1 | .5311 | .5311 | 3.64 |
| | Residual | 1 | .1458 | .1458 | |
| For carotene | Aeration | 1 | .5570 | .5570 | 2785* |
| | Time | 1 | .0252 | .0252 | 126 |
| | Residual | 1 | .0002 | .0002 | |

L.S.D. Carotene = 0.1792 L.S.D. Vitamin A = 4.8511

[a]All studies carried out at room temperature (23° to 25°).

## General Discussion

From the six series of trials regarding the effects of temperature, different varieties of alumina, light, minerals, aeration, and addition of nonsaponifiable fraction of lipid on the chromatographic separation of vitamin A and carotene, it was found that there was no significant loss of either vitamin A or carotene when the nonsaponifiable fraction of oil was omitted. Of course, it should be mentioned that the vitamin A which was used in the present study was dissolved in oil so even though cottonseed oil was not added during this experiment, there was a small quantity of oil already present. This might be responsible for some protection. To eliminate the above factor the experiment was repeated with ANRC Vitamin A Reference Standard, which was stabilized in gelatin and free from oil. In this trial also it was found that there was no loss of carotene or vitamin A. Thus apparently the addition of nonsaponifiable fraction of oil has no stabilizing effect in the chromatographic separation of carotene and vitamin A under present conditions.

It was mentioned previously that, in regard to the effect of ultraviolet light, the results are in contrast to those cited in the literature. Smith et al. (1939), Little (1944), and Bolomey (1947) observed that ultraviolet light destroyed vitamin A and carotene, forming different oxidized products. According to Little (1944), absorption of ultraviolet radiation is characteristic of unsaturated organic linkages and conjugated systems in particular, irradiation causing shifts of these bonds. Vitamin A and carotene, both having conjugated double bonds, are labile to destruction. In the present study there was no significant destruction, possibly due to the fact that radiations used were not so powerful as to cause these shifts of bonds; moreover, the glass apparatus used for chromatography no doubt absorbed some of the radiation.

It was observed that there were significant losses of vitamin A and carotene due to the effects of heat, strong light and acidic alumina. As previously mentioned, different isomerization products may be produced, as observed by various workers, accounting for some of the losses found.

The losses of vitamin A and carotene due to the effect of minerals (Cu, Fe) and effect of air may be due to oxidative destructions. These minerals may react as catalytic agents in causing destruction or oxidation. Vitamin A and carotene have several double bonds. Numerous chemical changes may take place, such as cyclization, double bond migration, or oxygen bridge formation at different points of unsaturation. Thus a number of transformations and degradation products may be formed, depending upon the particular point of reaction. Also, some of the vitamin A may be adsorbed tightly on certain of the columns, causing loss.

The losses observed under the above mentioned conditions may have taken place due to non-oxidative changes of the substances during chromatography. As previously observed by Gillam and El Ridi (1935), carotene and vitamin A isomerized on both alumina and calcium hydroxide columns. Meunier *et al.* (1951) observed also that when *trans* β-carotene and *trans* lycopene in petroleum ether were left in contact with alumina at room temperature, they changed to the *cis* form.

Thus heat, light, acidic alumina, minerals and aeration are factors which caused significant losses in the chromatographic separation of vitamin A and carotene on alumina. The addition of nonsaponifiable fraction of oil had no significant effect. The losses in vitamin A and carotene may be due to actual destruction, isomerization, oxidation, tight adsorption on the column, or various unknown reactions which are yet to be identified.

## SUMMARY

This study was undertaken to find the effects of: (1) temperature, (2) using neutral, basic and acidic aluminas, (3) using strong incandescant and ultraviolet lights, (4) addition of nonsaponifiable extract of lipid, (5) addition of minerals to alumina, and (6) passing air through the column, on the chromatographic separation and recovery of vitamin A.

The method used in this study was that in Methods of Analysis, A.O.A.C. (1960), with only minor changes.

It was apparent that at lower temperatures, that is, at $0^{\circ}$, $13^{\circ}$ and $23^{\circ}$, the carotenoids were eluted more slowly than at higher temperatures. The loss of carotene and vitamin A at $38^{\circ}$, as compared to $0^{\circ}$, $13^{\circ}$ and $23^{\circ}$, was statistically significant at the .01 level.

It was found that there was loss of carotene and vitamin A on the acidic alumina at $23^{\circ}$ and $38^{\circ}$. The loss of vitamin A was significant at the .05 level and that of carotene was significant at the .01 level. The results obtained using basic alumina, as compared to neutral alumina, were almost the same at $0^{\circ}$ and $23^{\circ}$.

It was found that the addition of nonsaponifiable extract of oil had no stabilizing effect on carotene or vitamin A and the results obtained with or without addition of nonsaponifiable fraction of oil were similar.

The loss of carotene under strong incandescant light was significant at the .05 level. The vitamin A lost under the same conditions was significant at the .01 level.

There was no significant loss of vitamin A or carotene under the effect of ultraviolet lights in comparison to ordinary light. This is possibly due to the fact that the radiations used were not sufficiently strong enough

to cause destruction.

The loss in carotene due to the presence of .001 percent copper and .01 percent iron in chromatographic alumina was significant at the .05 level only in diluted solution. The loss of vitamin A was significant at the .05 level also only in the dilute solution in the presence of .01 percent iron in the alumina.

When the alumina was aerated by laboratory air for 15 minutes or for one-half hour, there was not a significant loss of carotene or vitamin A. When prior to chromatography dry air was passed through alumina for 15 minutes or one-half hour, there was a significant loss of both carotene and vitamin A ($P = .05$). When laboratory air was passed through the column, vitamin A was eluted faster, and when dry air was passed through the column, the vitamin A was held more tightly on the column and approximately double the amount of 15 percent acetone in hexane was required for complete elution. It was observed further that when dry air was drawn through the column the moisture content of the alumina decreased, and when laboratory air was drawn through the column the moisture content increased. This may explain the rapid elution when atmospheric air was passed and tight adsorption when the column was aerated with dry air. Tight adsorption on the column may cause some loss of vitamin A on alumina preconditioned with dry air.

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# APPENDIX

# APPENDIX

## Sample Calculation of Analysis of Variances

Analysis of variance for effect of temperature on the chromatographic separation of vitamin A:

Percent deviation on vitamin A from results at room temperature (23°)[1] taken as standard.

| Temperatures | Vitamin concentrations | |
|---|---|---|
| | $C_1$ | $C_2$ |
| 0° | .8422[2] | 1.1111[2] |
| 13° | .1927[2] | -0.2670[2] |
| 38° | -3.6641[2] | -3.4588[2] |

[1]Average of eight observations at 23°C.

[2]Average of eight observations.

Ten added to each percent deviation (to remove negative numbers):

| Temperature | $C_1$ | $C_2$ | Total | | Average |
|---|---|---|---|---|---|
| 0°C | 10.8422 | 11.1111 | 21.9533 | $Temperature_1$ | 10.97605 |
| 13°C | 10.1927 | 9.7330 | 19.9257 | $Temperature_2$ | 9.96285 |
| 38°C | 6.3359 | 6.5412 | 12.8771 | $Temperature_3$ | 6.43855 |
| Total | 27.3708 | 27.3853 | 54.7561 | | |

T' (uncorrected total S.S.) = $(10.8422)^2 + \ldots\ldots + (6.5412)^2 = 522.5632$

I (correction factor) = $\frac{(54.7561)^2}{6} = 499.7051$

Temperature' (uncorrected temperature S.S.) = $\frac{(Temp_1)^2 + (Temp_2)^2 + (Temp_3)^2}{2}$

$= 522.4003$

Concentration' (uncorrected concentration S.S.) = $\frac{(Conc_1)^2 + (Conc_2)^2}{3}$

$= 499.7051$

Analysis of Variance

| Source | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Temperature (Temp'-1) | 2 | 22.6952 | 11.3476 | $\frac{11.3476}{0.0814} = 139.5769^{**}$ |
| Concentration (Conc'-1) | 1 | 0 | 0 | |
| Residual (T'-1-Temp-Conc) | 2 | 0.1629 | 0.0814 | |

** Significant at .01 level

$$L.S.D. = t_{05,2} \cdot \sqrt{\frac{2(.0814)}{2}} = (4.303) \cdot (.2853) = 1.2276$$

Table of Difference:

| Temperature | $\bar{x}$ | $\bar{x}$ - 6.43855 | $\bar{x}$ - 9.96285 |
|---|---|---|---|
| $0^{o}C$ | 10.97605 | $4.53750^{**}$ | 1.01320 |
| $13^{o}C$ | 9.96285 | 3.52430** | |
| $38^{o}C$ | 6.43855 | | |

# SOME FACTORS AFFECTING CHROMATOGRAPHIC SEPARATION OF VITAMIN A ON ALUMINA

by

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The effects of the following factors on the chromatographic separation and recovery of vitamin A and carotene on alumina were studied: (1) temperature, (2) using neutral, basic and acidic aluminas, (3) radiation with incandescent and ultraviolet lights, (4) addition of nonsaponifiable extract of lipid, (5) addition of minerals to the alumina, (6) preconditioning the column by passing air through it.

The method used in this experiment was essentially the same as given by A.O.A.C. (1960), with only minor changes.

In the temperature study, it was observed that the carotenoids were eluted more slowly at $0^{\circ}$, $13^{\circ}$, and $23^{\circ}$ than at a higher temperature ($38^{\circ}$). There was a loss of carotene and vitamin A at $38^{\circ}$ which was statistically significant ($P = .01$).

There was a significant ($P = .05$) loss of vitamin A on acidic alumina at $23^{\circ}$ and $38^{\circ}$, and of carotene ($P = .01$) at the same temperatures. There was no loss of either vitamin A or carotene using basic alumina, as compared to neutral alumina.

The results obtained with or without addition of nonsaponifiable fraction of oil were similar, and it appeared that addition of nonsaponifiable extract of oil had no stabilizing effect on carotene or vitamin A.

The effect of strong incandescent light was found to cause significant loss of carotene ($P = .05$), and vitamin A ($P = .01$). Ultraviolet lights did not cause significant loss of vitamin A or carotene. This was probably due to the fact that the radiations used were not strong enough to have measurable effect.

The presence of .001 percent copper or .01 percent iron in the chromatographic alumina caused significant loss in carotene ($P = .05$) only in the

dilute solution. The presence of .01 percent iron in alumina also caused a significant loss of vitamin A ($P = .05$) in the dilute solution.

There was no significant loss of carotene or vitamin A when atmospheric air was passed through the alumina for 15 minutes or one-half hour, but when the alumina was aerated by dry air for 15 minutes or one-half hour prior to chromatography, there was a significant loss of both carotene and vitamin A ( $P = .05$). Vitamin A was eluted rapidly when the alumina was aerated by atmospheric air, whereas it was held more tightly on the column when dry air was passed through alumina prior to chromatography. From the moisture determinations it was found that alumina preconditioned by dry air decreased in moisture content, whereas the alumina preconditioned by atmospheric air increased in moisture content, which possibly explains the tight adsorption of vitamin in the former case and quick elution in the latter. Tight adsorption on the column may cause some of the loss of vitamin A on alumina, as mentioned above.